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## Ammonia Production and Its Possible Role as a Mediator of Communication for *Debaryomyces hansenii* and Other Cheese-Relevant Yeast Species

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### ABSTRACT

Ammonia production by yeasts may contribute to an increase in pH during the ripening of surface-ripened cheeses. The increase in pH has a stimulatory effect on the growth of secondary bacterial flora. Ammonia production of single colonies of *Debaryomyces hansenii*, *Saccharomyces cerevisiae*, *Yarrowia lipolytica*, and *Geotrichum candidum* was determined on glycerol medium (GM) agar and cheese agar. The ammonia production was found to vary, especially among yeast species, but also within strains of *D. hansenii*. In addition, variations in ammonia production were found between GM agar and cheese agar. Ammonia production was positively correlated to pH measured around colonies, which suggests ammonia production as an additional technological parameter for selection of secondary starter cultures for cheese ripening. Furthermore, ammonia appeared to act as a signaling molecule in *D. hansenii* as reported for other yeasts. On GM agar and cheese agar, *D. hansenii* showed ammonia production oriented toward neighboring colonies when colonies were grown close to other colonies of the same species; however, the time to oriented ammonia production differed among strains and media. In addition, an increase of ammonia production was determined for double colonies compared with single colonies of *D. hansenii* on GM agar. In general, similar levels of ammonia production were determined for both single and double colonies of *D. hansenii* on cheese agar.

**Key words:** *Debaryomyces hansenii*, ammonia production, signaling, cheese

### INTRODUCTION

Surface-ripened cheeses are characterized by a ripening mediated by a smear consisting of a mixed popula-

tion of yeasts and surface bacteria, which contributes to the development of cheese flavor and texture due to its proteolytic and lipolytic activities (Brennan et al., 2004). The predominant yeast species observed during cheese ripening is *Debaryomyces hansenii*; however, strains of *Saccharomyces cerevisiae*, *Yarrowia lipolytica*, and *Geotrichum candidum* as well as a number of other yeast species have been reported (Fleet, 1990; Eliskases-Lechner and Ginzing, 1995b; Jakobsen and Narvhus, 1996; Corsetti et al., 2001; Petersen et al., 2002). The growth of the less acid-tolerant bacteria including *Brevibacterium linens*, *Corynebacterium* spp., *Arthobacter* spp., *Micrococcus* spp., and *Staphylococcus* spp. is dependent on an increase in pH from about 5.0 to 5.8 or even >6 for some bacteria on the cheese surface (Seiler, 1986; Eliskases-Lechner and Ginzing, 1995a; Valdés-Stauber et al., 1997). Generally, it is accepted that degradation of lactate in aerobiose to CO<sub>2</sub> and H<sub>2</sub>O by the yeasts leads to an increase of pH (Eliskases-Lechner and Ginzing, 1995b; Leclercq-Perlat et al., 1999), but production of alkaline metabolites such as ammonia may also be of importance (Brennan et al., 2004). However, the influence of ammonia production on the increase of pH has not been investigated to the same extent as lactate metabolism.

Quorum sensing is a well-known phenomenon by which microorganisms sense and respond to cell density-dependent signaling molecules (Miller and Bassler, 2001; Fuqua and Greenberg, 2002). Initial studies on quorum sensing have mostly focused on bacteria, and the phenomenon may be involved in growth of pathogenic and spoilage bacteria in foods such as meat products (Christensen et al., 2003; Bruhn et al., 2004), milk (Jay et al., 2003), and vegetables (Rasch et al., 2005). However, little is known about the role of quorum sensing of yeasts present in foods. In yeasts, quorum sensing has been described before; for example, in coordination of metabolism in *S. cerevisiae* at high cell densities with acetaldehyde as the signaling molecule (Richard et al., 1996); in stimulation of meiosis and sporulation in *S. cerevisiae* with bicarbonate as the signaling molecule

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(Hayashi et al., 1998; Ohkuni et al., 1998); and in prevention and stimulation of mycelial development in *Candida albicans* with farnesol and tyrosol as the respective signaling molecules (Hornby et al., 2001; Chen et al., 2004). Furthermore, ammonia has been reported to be involved in development and survival of neighboring colonies on agar plates (Palkova et al., 1997).

Produced ammonia has been found to be transmitted to neighboring yeast colonies, which responded by enhancing their own ammonia production, indicating that ammonia is a signaling molecule (Palkova et al., 1997). An increase in ammonia production has been found to lead to an alternative and more economical metabolism resulting in cell survival (Palkova and Forstova, 2000; Palkova et al., 2002; Palkova and Vachova, 2003). In addition, ammonia signaling typically results in asymmetric colonies forced to grow into the free space rich in nutrients. Several findings indicate that ammonia production involves amino acids released from the cellular stock or from protein degradation (Palkova et al., 1997; Zikanova et al., 2002). Ammonia has been reported as a general signaling molecule in yeasts. However, the role of ammonia as a signaling molecule involved in development and survival of colonies of *D. hansenii* highly relevant for cheese ripening has not yet been investigated.

In this study, the role of ammonia in the increase of pH on cheese surfaces was investigated by measurement of ammonia production of single colonies of different yeast species important for cheese ripening. Furthermore, it has been investigated whether strains of *D. hansenii* use ammonia for signaling and, thus, coordination of growth on both synthetic and cheese media.

## MATERIALS AND METHODS

### Strains and Culture Conditions

Three strains of *Debaryomyces hansenii*: D 18335, a dairy isolate (Petersen et al., 2001, 2002); MD 02 (Arla Innovation, Brabrand, Denmark); and the type strain of *D. hansenii* var. *hansenii* CBS 767 (Centraalbureau voor Schimmelcultures, Baarn and Delft, the Netherlands) were used in the present study. Furthermore, *Saccharomyces cerevisiae* D 7 (Gorgonzola isolate, University of Copenhagen, Denmark), type strains of *Yarrowia lipolytica* (CBS 2075, Centraalbureau voor Schimmelcultures), and *Geotrichum candidum* (CBS 615.84, Centraalbureau voor Schimmelcultures) were included in the study.

Yeast cultures were maintained at  $-80^{\circ}\text{C}$  in yeast glucose peptone (YGP) broth [per liter: 5 g of yeast extract (Difco Laboratories, Detroit, MI), 10 g of glucose (Merck, Darmstadt, Germany), and 5 g of Bacto peptone (Difco Laboratories), pH 5.6] containing 20% (vol/vol)

glycerol (Merck). Yeast cultures were propagated in 2 steps; first, YGP (25 mL) was inoculated with 1 mL of freeze culture and incubated for 48 h at  $25^{\circ}\text{C}$  with shaking at 120 rpm. Cells were counted, and 100 mL of YGP was inoculated with  $1 \times 10^6$  cells per mL, and incubated for 48 h under the same conditions as for the first propagation step, before the cultures were spotted onto agar plates.

### Determination of Ammonia Production

Ammonia production was determined on glycerol medium (GM) agar [per liter: 10 g of yeast extract (Difco Laboratories), 3% (vol/vol) glycerol (Merck), 30 mM  $\text{CaCl}_2$  (Merck), and 20 g of agar (Difco Laboratories)] and on cheese agar. Cheese agar was prepared by autoclaving 20 g of Bacto agar (Difco) in 500 mL of demineralized water for 20 min at  $121^{\circ}\text{C}$ , and then mixing it with 200 g of unsalted Danbo cheese and 12.5 g of trisodium citrate dihydrate (Merck) cooked in 400 mL of demineralized water for 1 h before use. To determine the influence of free amino acids on ammonia production, 0.5% casamino acids (Difco Laboratories) was added to the cheese agar. Before autoclaving, 0.01% (wt/vol) of the pH indicator bromocresol purple (Sigma, St. Louis, MO) was added to both GM agar and cheese agar. The pH of both media was adjusted to 5.0, resulting in a dark yellow color of the GM agar and a bright green color of the cheese agar. Production of alkaline metabolites including ammonia changed the pH indicator to purple, whereas production of acidic metabolites changed the pH indicator to yellow. For determination of ammonia production of single colonies, 1 spot of 25  $\mu\text{L}$  of yeast culture ( $1 \times 10^7$  cells) was spotted on the agar plates, whereas for determination of ammonia as a signaling molecule, 2 spots of 25  $\mu\text{L}$  of yeast culture ( $1 \times 10^7$  cells) were spotted 1.5 cm apart on the agar plates, which subsequently were inverted. For absorption of volatile compounds produced by the growing colonies, a trap containing 200  $\mu\text{L}$  of 2% (wt/vol) HCl was placed under the colonies. Plates were incubated at  $25^{\circ}\text{C}$  from d 0 to 8. Every 24 h, the 200  $\mu\text{L}$  of HCl with absorbed volatile compounds was transferred to a 1.5-mL semimicro cuvette (Brand GmbH and Co, Wertheim, Germany) and 800  $\mu\text{L}$  of Nessler's reagent (Bie and Berntsen, Rødovre, Denmark) was added. After 30 min of incubation, the optical density ( $\text{OD}_{400\text{nm}}$  and  $\text{OD}_{500\text{nm}}$ ) was measured using a Shimadzu UV-1201 spectrophotometer (SpectroChrom, Brøndby, Denmark). A standard curve using  $\text{NH}_4\text{Cl}$  (Merck) was produced and used for calculation of the ammonia concentration. According to Barnes and Sugden (1990), linearity between  $\text{OD}_{400\text{nm}}$  and the concentration of ammonia was determined in the range of 5 to 20 ppm, whereas

linearity between OD<sub>500nm</sub> and the concentration of ammonia was determined in the range of 20 to 100 ppm. Colony diameters were measured and colony areas were calculated. The ammonia production of colonies was calculated as ppm/h per cm<sup>2</sup>. All trials were performed in triplicate.

### pH Measurement

Measurements of pH around yeast colonies were performed by the use of a surface electrode (InLab 426, Mettler-Toledo, Glostrup, Denmark) connected to a pH meter (1120, Mettler-Toledo). Adjustment of the electrode was performed with buffers with pH 4.01 and 7.00 (Radiometer, Brønshøj, Denmark).

### Determination of Lactate Degradation

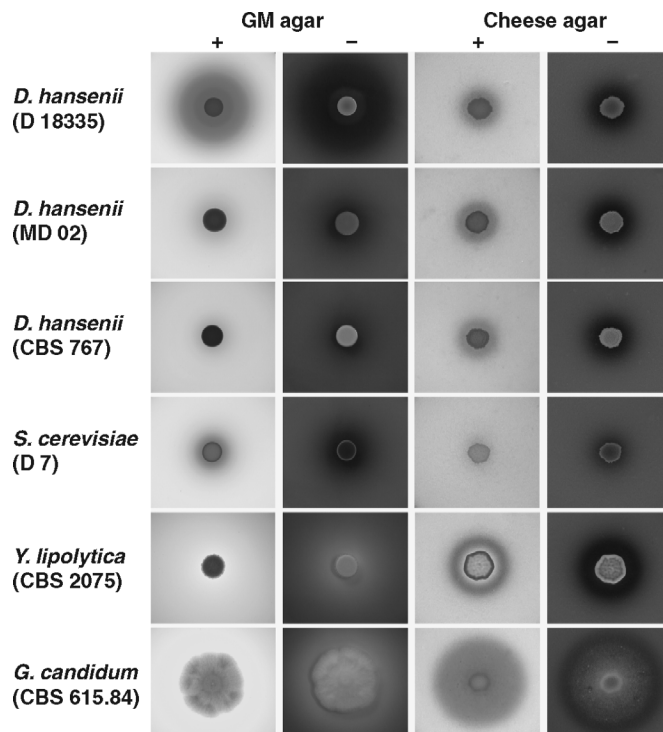
Plugs with an area of 1 cm<sup>2</sup>, which corresponds to the area of the pH electrode, were taken out around single colonies on cheese agar. Four plugs were homogenized with 1 mL of water for 2 min using a stomacher. One milliliter of 1.7 M TCA was added, and the suspension was homogenized for another 2 min, and then incubated at 25°C for 1 h. After incubation, the suspension was homogenized for another 2 min and filtered through a Millipore filter (0.2 µm, Millipore, Billerica, MA). Lactate was measured by HPLC (HP series 1100, Hewlett-Packard, Palo Alto, CA) using a MicroGuard cation H cartridge followed by an Aminex 87 H column (both from BioRad Laboratories, Hercules, CA). Lactate concentration was calculated as grams of lactate per gram of cheese agar.

### Determination of Casein Degradation

Casein degradation was investigated on casein agar [per liter: 10 g of Hammerstein casein (Merck), 0.3 g of Ca(OH)<sub>2</sub> (Merck), 0.2 g of CaCl<sub>2</sub>·2H<sub>2</sub>O (Merck), and 15 g of agar (Difco Laboratories), pH 5.8]. One spot of 25 µL of yeast culture (1 × 10<sup>7</sup> cells) was spotted on casein agar, and casein degradation was determined as clearing zones around colonies.

### Photography

Colonies were photographed with a digital camera (EOS350D, Canon Inc., Tokyo, Japan) with and without transillumination. Photos with transillumination were used to visualize the orientation of ammonia production, whereas photos without transilluminations were used to visualize changes in colony morphology.



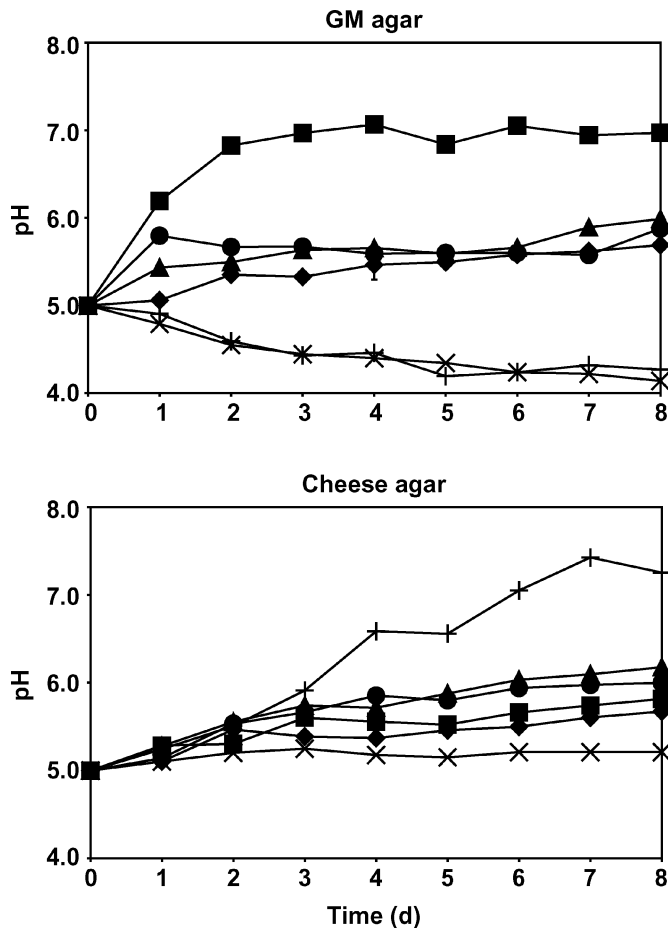
**Figure 1.** Ammonia production of single colonies of *Debaryomyces hanseii* (strains D 18335, MD 02, and CBS 767), *Saccharomyces cerevisiae* (D 7), *Yarrowia lipolytica* (CBS 2075), and *Geotrichum candidum* (CBS 615.84) determined on glycerol medium (GM) agar and cheese agar. Colonies were photographed after 4 d of incubation. Photos with transillumination were prepared to visualize changes in ammonia production (+), whereas photos without transillumination were prepared to visualize changes in colony morphology (-). Color figure available at <http://jds.fass.org/content/vol90/issue11/>.

## RESULTS

### Ammonia Production for Single Colonies

Ammonia production was followed by color changes of the pH indicator bromocresol purple around single colonies of *D. hanseii* (D 18335, MD 02, and CBS 767), *S. cerevisiae* (D 7), *Y. lipolytica* (CBS 2075), and *G. candidum* (CBS 615.84) on glycerol medium (GM) agar and cheese agar, respectively (Figure 1). On GM agar, a shift from dark yellow to purple was observed for *D. hanseii* (D 18335, MD 02, and CBS 767) and *S. cerevisiae* (D 7), indicating that these yeast strains produced ammonia, whereas a shift from dark yellow to bright yellow was observed for *Y. lipolytica* (CBS 2075) and *G. candidum* (CBS 615.84), indicating that these yeast strains did not produce ammonia. The most intense purple color was observed for *D. hanseii* (D 18335). On cheese agar, a shift from bright green to purple indicating ammonia production was observed for all the investigated yeasts. The most intense purple color was observed for *Y. lipolytica* (CBS 2075).





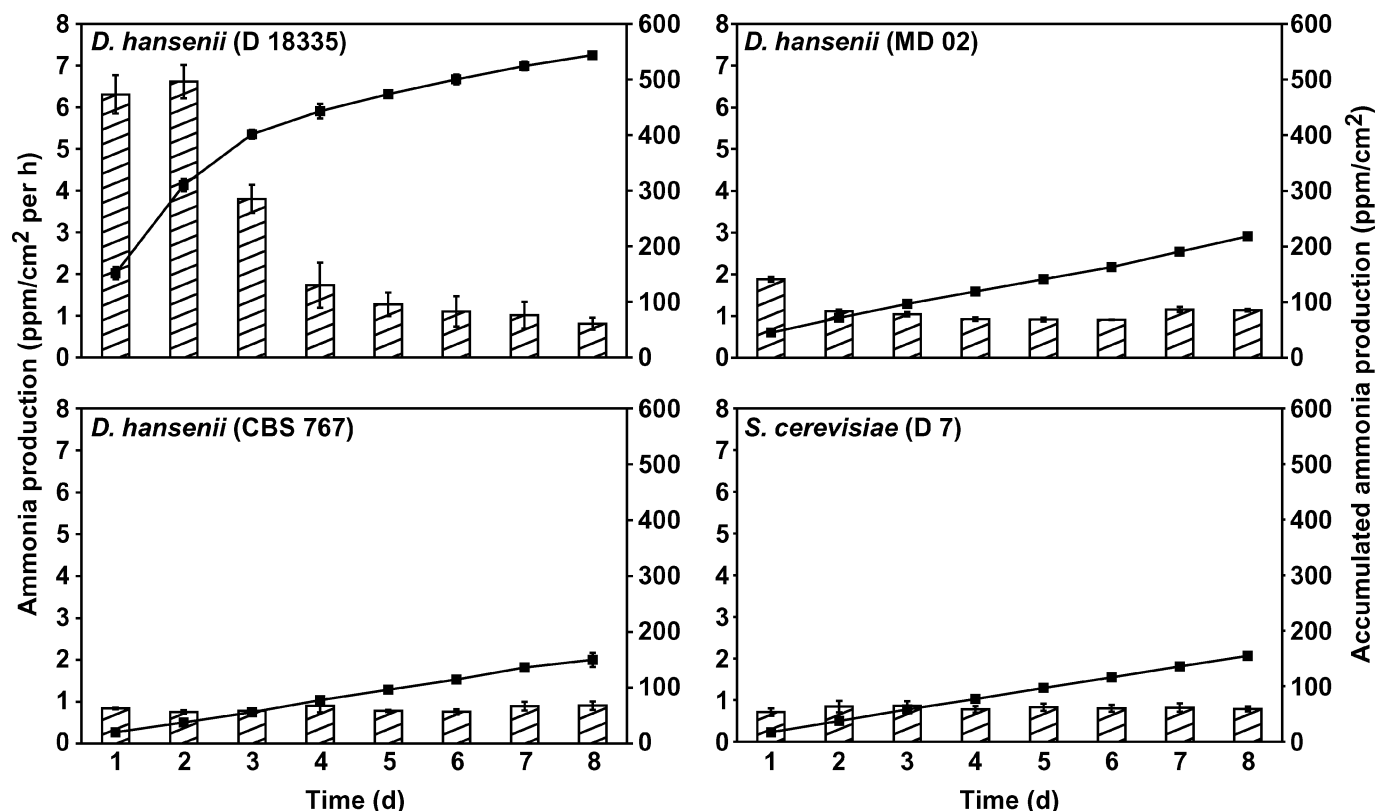
**Figure 2.** Measurement of pH around single colonies of *Debaryomyces hansenii* (strains D 18335, MD 02, and CBS 767), *Saccharomyces cerevisiae* (D 7), *Yarrowia lipolytica* (CBS 2075), and *Geotrichum candidum* (CBS 615.84) determined on glycerol medium (GM) agar and cheese agar. Yeast strains: ■ = *D. hansenii* (D 18335); ● = *D. hansenii* (MD 02); ▲ = *D. hansenii* (CBS 767); ◆ = *S. cerevisiae* (D 7); + = *Y. lipolytica* (CBS 2075), and × = *G. candidum* (CBS 615.84).

The pH was measured around yeast colonies to verify the effects observed by changes of the pH indicator (Figure 2). On both GM agar and cheese agar, a good correlation between the color changes of bromocresol purple and pH measurements was observed. On GM agar, the greatest increase of pH was observed around single colonies of *D. hansenii* (D 18335), for which pH increased from 5.0 to 6.8 during the first 2 d, and remained constant at this value during the rest of the 8 d period. For *D. hansenii* (MD 02 and CBS 767) and *S. cerevisiae* (D 7), pH increased from 5.0 to an average value of 5.4 during d 1 and 2, and remained at this value. For *Y. lipolytica* (CBS 2075) and *G. candidum* (CBS 615.84), pH slightly decreased from 5.0 to 4.3 and 4.1, respectively, during the investigated period. On cheese agar, the greatest increase in pH was observed around single colonies of *Y. lipolytica* (CBS 2075), for

which pH slightly increased to 7.3 on d 8. The pH around single colonies of *D. hansenii* (CBS 767 and MD 02) increased respectively to 6.2 and 6.0 on d 8, whereas pH around single colonies of *D. hansenii* (D 18335) and *S. cerevisiae* (D 7) increased from 5.0 to slightly lower pH values (5.8 and 5.7, respectively). No changes in pH were observed around single colonies of *G. candidum* (CBS 615.84).

Ammonia production of single colonies of the included yeast strains was determined on both GM agar and cheese agar by Nessler's reagent. On GM agar, ammonia production was determined for *D. hansenii* (D 18335, MD 02, and CBS 767) and *S. cerevisiae* (D 7), whereas no ammonia production was detected for *Y. lipolytica* (CBS 2075) and *G. candidum* (CBS 615.84) (Figure 3). The greatest ammonia production on GM agar was determined for *D. hansenii* (D 18335). High ammonia production was noted particularly during the first 2 d, when production of 6.3 and 6.6 ppm/h per cm<sup>2</sup> was determined, respectively. *Debaryomyces hansenii* (MD 02 and CBS 767) and *S. cerevisiae* (D 7) produced similar levels of ammonia (between 0.72 and 1.9 ppm/h per cm<sup>2</sup>) during the investigational period. On cheese agar, all the investigated strains produced ammonia (Figure 4), particularly *Y. lipolytica* (CBS 2075), which produced very high levels on cheese agar (unlike GM agar, in which no ammonia production was observed). The ammonia production of *Y. lipolytica* (CBS 2075) was 4.4 ppm/h per cm<sup>2</sup> on d 1, increased to 30 ppm/h per cm<sup>2</sup> on d 5, before it decreased to 9.5 ppm/h per cm<sup>2</sup> on d 8. *Debaryomyces hansenii* (MD 02 and CBS 767) produced ammonia levels between 0.87 and 1.8 ppm/h per cm<sup>2</sup>, whereas *D. hansenii* (D 18335) and *S. cerevisiae* (D 7) produced slightly lower levels of ammonia (between 0.69 and 0.93 ppm/h per cm<sup>2</sup>). *Geotrichum candidum* (CBS 615.84) produced very low ammonia levels (between 0.042 and 0.30 ppm/h per cm<sup>2</sup>); however, the low value was primarily because of the increased colony size observed for this yeast strain. Without taking colony area into consideration, *G. candidum* (CBS 615.84) produced similar levels of ammonia as *D. hansenii* (D 18335) and *S. cerevisiae* (D 7) on cheese agar (results not shown).

Ammonia production for all strains of *D. hansenii* increased on cheese agar with the addition of 0.5% (wt/vol) casamino acids (Figure 5). No significant differences could be observed among strains investigated. By d 1, an increase in ammonia production could be observed [465% in average for *D. hansenii* (D 18335, MD 02, and CBS 767)] compared with cheese agar without addition of amino acids. On d 8, ammonia production was increased [408% on average for *D. hansenii* (D 18335, MD 02, and CBS 767)], even though the level was slightly decreased compared with that on d 5 [585%



**Figure 3.** Ammonia production (bars) of single colonies of *Debaryomyces hansenii* (strains D 18335, MD 02, and CBS 767), *Saccharomyces cerevisiae* (D 7), *Yarrowia lipolytica* (CBS 2075), and *Geotrichum candidum* (CBS 615.84) determined on glycerol medium (GM) agar by Nessler's reagent; *Y. lipolytica* (CBS 2075) and *G. candidum* (CBS 615.84) did not produce ammonia on GM agar. The accumulated ammonia production is shown in curves.

on average for *D. hansenii* (D 18335, MD 02, and CBS 767)].

### Lactate Degradation

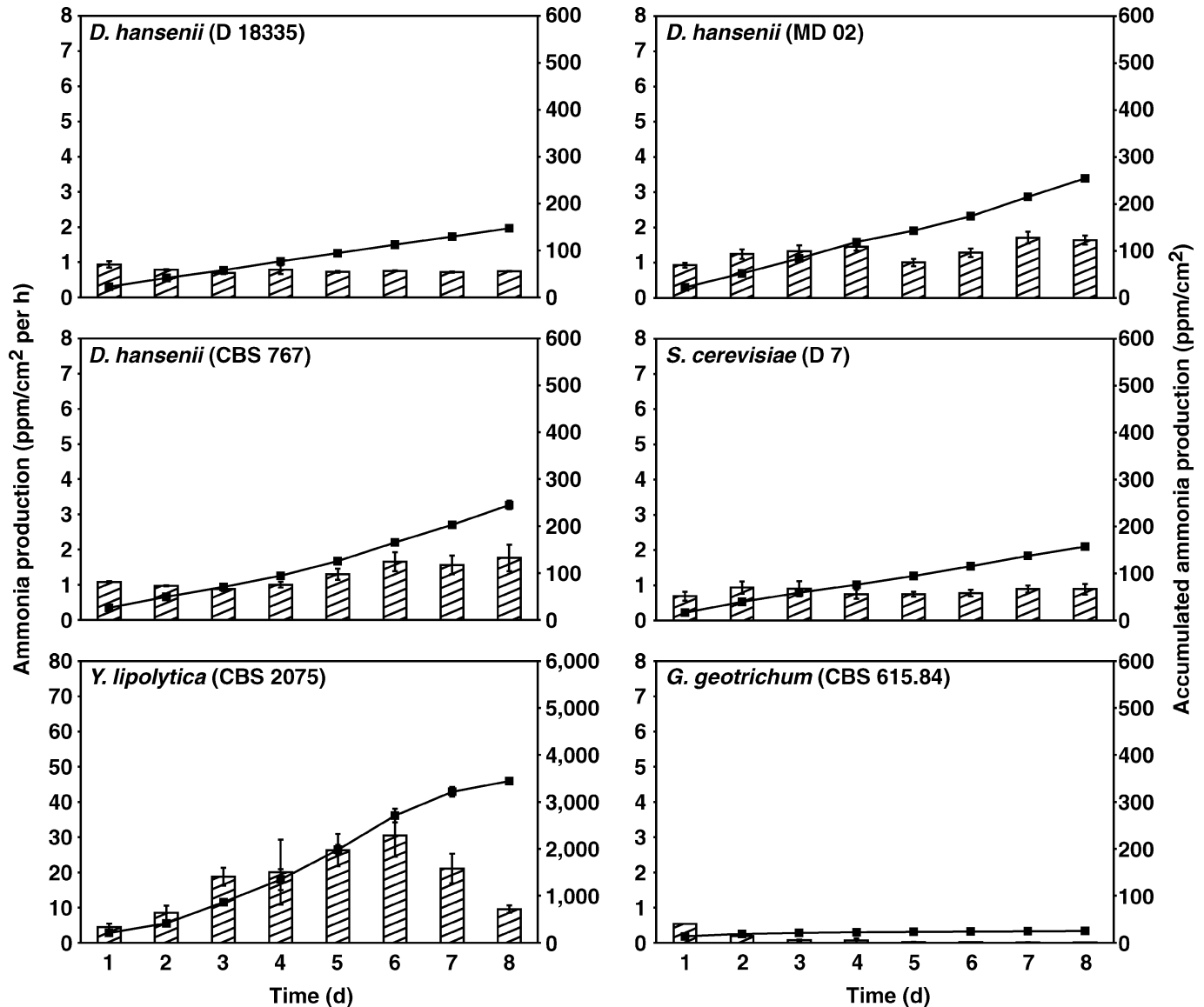
Lactate degradation was determined in plugs excised around single colonies on cheese agar. For *D. hansenii* (D 18335, MD 02, and CBS 767), *S. cerevisiae* (D 7), and *Y. lipolytica* (CBS 2075), the level of lactate slightly decreased from 0.2 to 0.1 g of lactate per 100 g of cheese agar during the 8 d (data not shown). No significant lactate degradation was shown in plugs taken out around the colonies of the filamentous *G. candidum* (CBS 615.84).

### Ammonia Production for Neighboring Colonies

The use of ammonia as a signaling molecule was investigated for *D. hansenii* (D 18335, MD 02, and CBS 767). Ammonia production was followed, when 2 colonies were spotted 1.5 cm apart on both GM agar and cheese agar (Figure 6). On GM agar and cheese agar, *D. hansenii* (D18335, MD 02, and CBS 767) showed

ammonia production oriented toward neighboring colonies; however, the time to oriented ammonia production differed between strains and media. On GM agar, significantly oriented ammonia production between double colonies of *D. hansenii* (D 18335) was observed after 12 h, whereas for *D. hansenii* (MD02 and CBS 767), it was observed after 16 and 20 h, respectively. Unlike *D. hansenii* (MD 02 and CBS 767), for which ammonia was primarily oriented toward neighboring colonies, *D. hansenii* (D 18335) produced ammonia in directions other than toward neighboring colonies. On cheese medium, oriented ammonia production between double colonies was observed later than on GM agar. On cheese medium, significant oriented ammonia production between double colonies of *D. hansenii* (MD 02) was observed after 28 h, whereas double colonies of *D. hansenii* (D 18335 and CBS 767) showed significantly oriented ammonia production after 36 h. Asymmetric growth of the double colonies expanding to the free area was observed for *D. hansenii* (D18335, MD 02, and CBS 767) on cheese agar, unlike on GM agar.

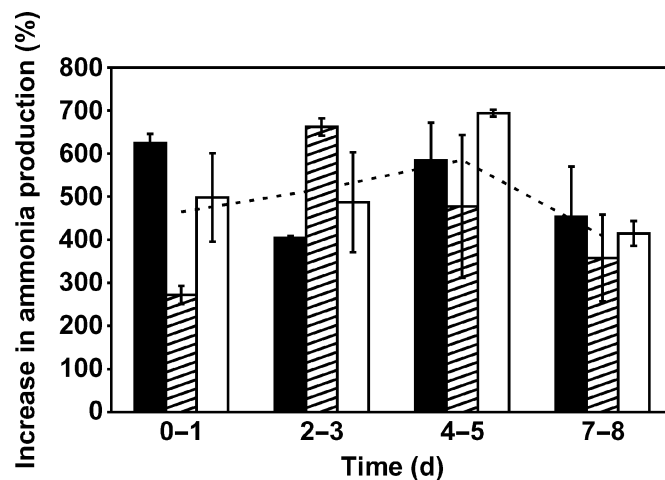
Ammonia production for double colonies of *D. hansenii* (D18335, MD 02, and CBS 767) was determined



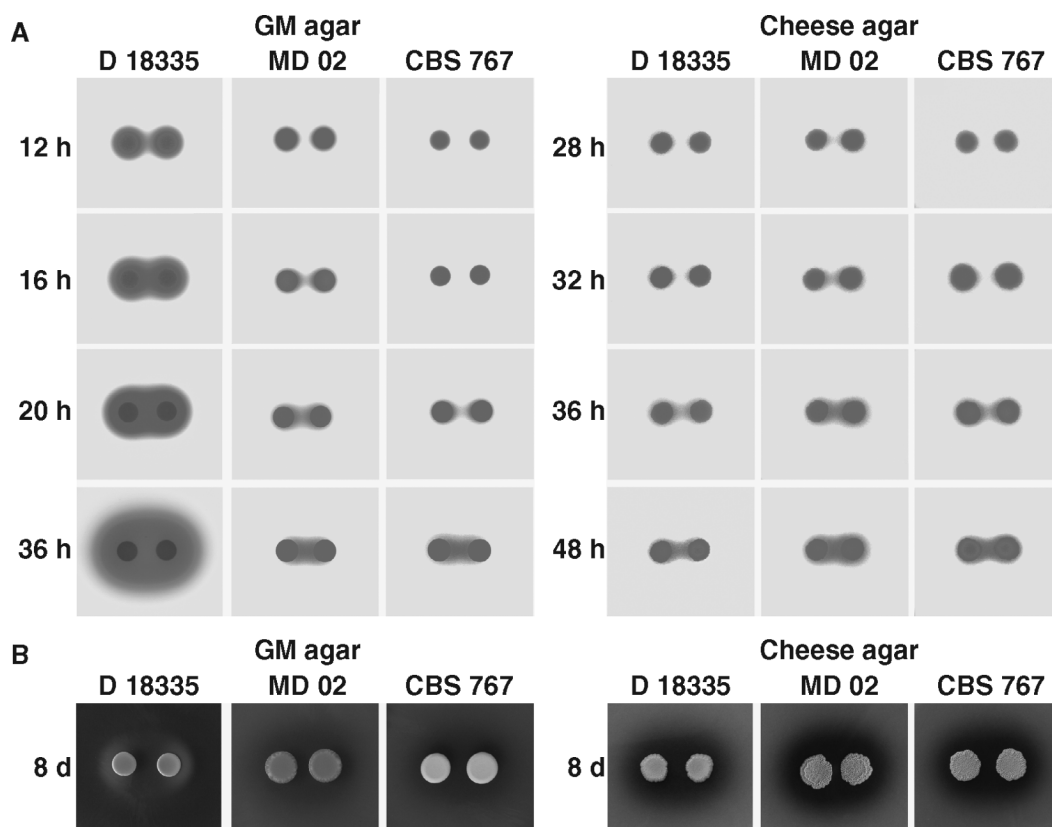
**Figure 4.** Ammonia production (bars) of single colonies of *Debaryomyces hanseii* (strains D 18335, MD 02, and CBS 767), *Saccharomyces cerevisiae* (D 7), *Yarrowia lipolytica* (CBS 2075), and *Geotrichum candidum* (CBS 615.84) determined on cheese agar by Nessler's reagent. The accumulated ammonia production is shown in curves. Note that the axes for the ammonia production and the accumulated ammonia production of *Y. lipolytica* (CBS 2075) are increased by a factor of 10.

by Nessler's reagent, and ammonia production per colony and the percentage increase or decrease in ammonia production for double colonies compared with single colonies were calculated (Figure 7). On GM agar, the ammonia production of double colonies was greater than that obtained for single colonies of any *D. hanseii* strain. Indeed, a significant increase in ammonia production of double colonies compared with single colonies was observed from d 3. For *D. hanseii* (D 18335), the increase in ammonia production was 27% on d 3, and continued to increase (164% on d 6 and 144% on d

8). For *D. hanseii* (MD 02), the increase of ammonia production was 27% on d 3, it continued to increase to 77% on d 6, and then it decreased to 17% on d 8. For *D. hanseii* (CBS 767), the increase in ammonia production was 52% on d 3, it continued to increase to 128% on d 6, and then it decreased to 77% on d 8. On cheese agar, an increase in the ammonia production for double colonies compared with single colonies was only seen for *D. hanseii* (MD 02) from d 4 to 6, especially on d 5, when an increase of 55% was obtained. Any increase in ammonia production for double colonies compared

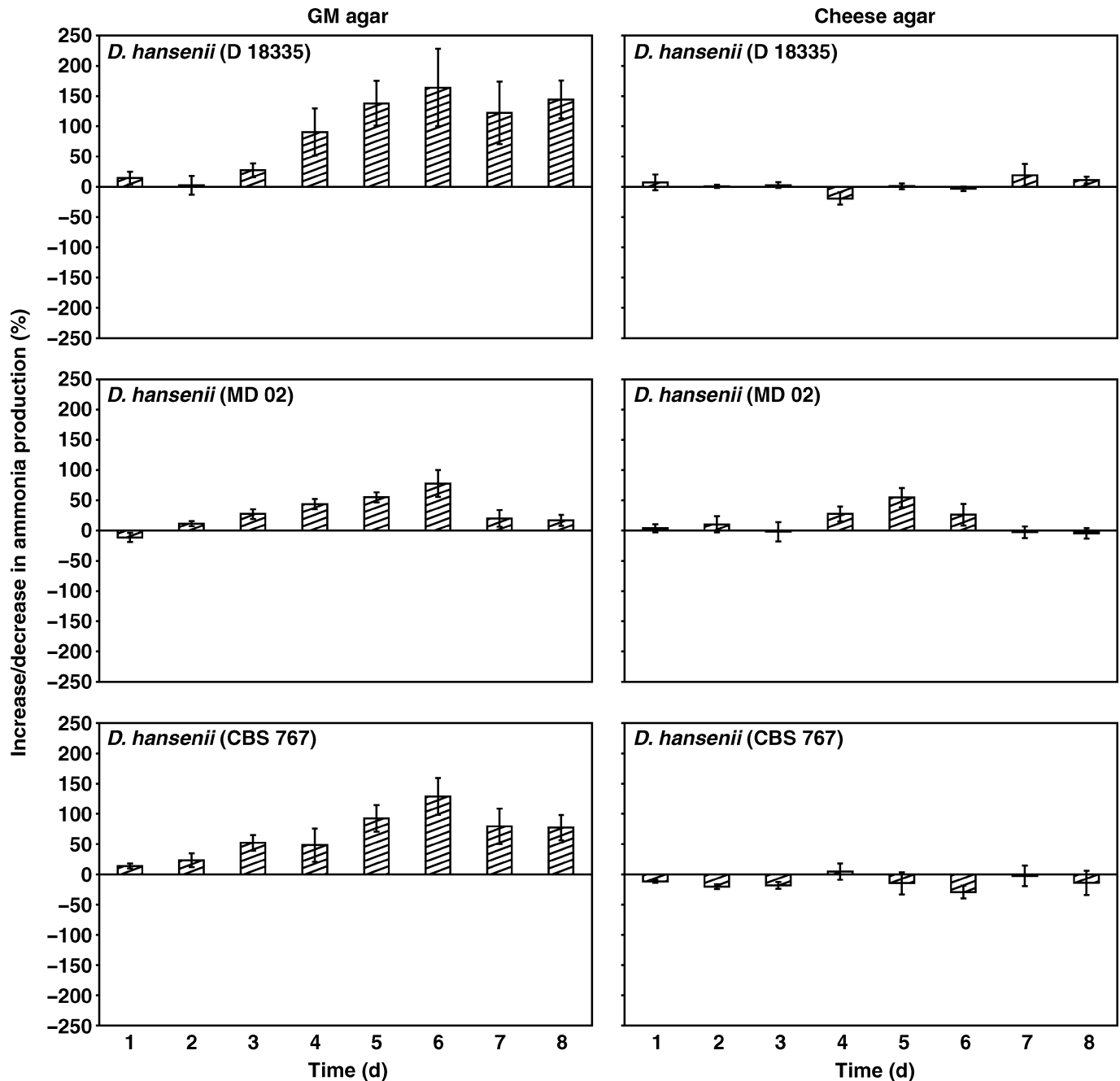


**Figure 5.** Increase in ammonia production (%) by addition of 0.5% (wt/vol) casamino acids to cheese agar for *Debaryomyces hansenii* D 18335 (black bars); *D. hansenii* MD 02 (gray bars); and *D. hansenii* CBS 767 (white bars). The dotted line shows the average increase in ammonia production (%) for *D. hansenii* (D 18335, MD 02, and CBS 767).



**Figure 6.** Ammonia production of double colonies of *Debaryomyces hansenii* (strains D 18335, MD 02, and CBS 767) on glycerol medium (GM) agar and cheese agar. Photos with transillumination (A) were prepared to visualize changes in ammonia production, whereas photos without transillumination (B) were prepared to visualize changes in colony morphology. Color figure available at <http://jds.fass.org/content/vol90/issue11/>.





**Figure 7.** Increases and decreases (%) in ammonia production of double colonies compared with single colonies for *Debaryomyces hansenii* (strains D 18335, MD 02, and CBS 767). The ammonia production for double colonies was calculated per colony.

with single colonies was observed for the other *D. hansenii* (D 18335 and CBS 767).

## DISCUSSION

Ammonia production of the investigated yeasts was found to vary between species, but also among strains of *D. hansenii*. In addition, differences in ammonia pro-

duction of the investigated yeasts were observed between GM agar and cheese agar. This is probably due to variations in the free amino acid content of the 2 media, because GM agar contains approximately 3 g/L of free amino acids supplied from the yeast extract, whereas cheese agar contains only 0.3 g/L of free amino acids (Ardö et al., 2002). Because only *Y. lipolytica* (CBS 2075) was able to degrade casein during the 8-d period

(results not shown), casein degradation would not have contributed to ammonia production in *D. hansenii* (D18335, MD 02, and CBS 767), *S. cerevisiae* (D 7), or *G. candidum* (CBS 615.84) on cheese agar. Ammonia production for *S. cerevisiae* has been reported to be highly dependent on the availability of amino acids (Palkova et al., 1997; Zikanova et al., 2002). This was further confirmed in the present study for *D. hansenii* by addition of casamino acids to cheese agar (Figure 5). Furthermore, differences in ammonia production may be influenced by the ability of the investigated yeast strains to grow on glycerol, the carbon source in GM agar, or lactate, one of the main carbon sources in cheese agar.

The results showed that yeasts found on cheese surfaces produced ammonia indicating that ammonia production, as suggested by Brennan et al. (2004), may play a role in the increase of pH on cheese surfaces. A correlation between an increase of pH and ammonia production was observed for all yeast strains on both GM agar and cheese agar, indicating that differences in ammonia production are mainly responsible for these differences in pH. Degradation of lactate has earlier been reported to play an important role in the increase of pH around colonies (Leclercq-Perlat et al., 1999). However, determination of lactate degradation in plugs excised around colonies grown on cheese agar showed that the lactate level decreased from 0.2 to 0.1 g per 100 g of cheese during the 8 d for all yeasts except of *G. candidum*. With respect to *G. candidum*, this study showed low levels of ammonia production (calculated per colony area) even though *G. candidum* is generally known for its deamination of amino acids resulting in ammonia production during cheese ripening (Hemme et al., 1982; Karahadian and Lindsay, 1987). In addition, no increase of pH around *G. candidum* colonies was determined. These observations may be biased due to the growth characteristics of *G. candidum* because ammonia is only involved in an increase of pH in the agar under the filamentous colony. Moreover, *G. candidum* is primarily used for flavor development, not to deacidify the cheese surface (Bockelmann et al., 2005). Based on the results obtained in the present study, ammonia production by yeasts may be taken into consideration as an additional technological parameter when starter cultures are selected for surface-ripened cheeses as previously described for other technological parameters such as NaCl tolerance (Gori et al., 2005) and adhesion (Mortensen et al., 2005).

Ammonia as a signaling molecule has been reported to be important for colony survival and development (Palkova et al., 1997). The signaling phenomenon is suspected to be general for yeasts. However, in this study, no ammonia production was determined for ei-

ther *Y. lipolytica* (CBS 2075) or *G. candidum* (CBS 615.84) on GM agar earlier used to determine ammonia as a signaling molecule, indicating that ammonia is not involved in signaling in these yeast species. For the first time, this study showed that ammonia is involved in signaling in strains of *D. hansenii*. For *D. hansenii* (D 18335, MD 02, and CBS 767), ammonia was found to be oriented toward neighboring colonies when grown 1.5 cm apart on both GM agar and cheese agar, indicating that colonies of *D. hansenii* use ammonia as a signaling molecule for coordination of growth as reported for other yeast species (Palkova et al., 1997). In addition, ammonia production was greater for double colonies compared with single colonies of *D. hansenii* (D 18335, MD 02, and CBS 767) on GM agar. However, on cheese medium, ammonia production was only found to be greater for double colonies of *D. hansenii* (MD 02) from d 4 to 6, even though a clearly oriented ammonia production could be observed on both GM agar and cheese agar. The deficiency in increased ammonia production on cheese agar may be because *D. hansenii* (D 18335, MD 02, and CBS 767) do not have sufficient proteolytic activity to degrade casein to free amino acids, which are responsible for ammonia production. Moreover, *D. hansenii* produces only endopeptidases (Klein et al., 2002). The cheese agar used will differ in several aspects from the environment on surface-ripened cheeses; for example, the composition of the microflora (and thus, the availability of different nitrogen sources) will be different. However, it is possible that ammonia production will increase for double colonies after d 8.

Ammonia production is related to the colony structure such as observed for *Candida mogii*, in which ammonia changed the colony morphology from smooth to ruffled (Palkova and Forstova, 2000). In this study, *D. hansenii* (MD 02 and CBS 767) (the greatest ammonia production on cheese agar) showed ruffled colony morphology, whereas *D. hansenii* (D 18335) (the lowest ammonia production on cheese agar) showed smooth colony morphology. Ruffled colony morphology may be associated with longer lifetime of colonies due to more efficient water fluxes and removal of waste products compared with smooth colonies (Palkova et al., 2002).

In conclusion, ammonia production varied among yeast species and among different strains of *D. hansenii* and was influenced by the substrates used. Ammonia production plays an important role in the increase of pH on cheese surfaces, and may be taken into consideration as a technological parameter when starter cultures are selected for surface-ripened cheeses. Furthermore, ammonia apparently functions as a signaling molecule in *D. hansenii* as reported for other yeast strains. New knowledge about how *D. hansenii* communicate in complex environments such as a cheese sur-

face will lead to increased information on how this important yeast species grows and proliferates in dairy products.

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